

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁴ : A61K 37/02, C07K 5/12, 7/06 C07K 7/08, 7/10	A1	(11) International Publication Number: WO 89/ 05150 (43) International Publication Date: 15 June 1989 (15.06.89)
---	----	--

(21) International Application Number: PCT/US88/04403

(22) International Filing Date: 8 December 1988 (08.12.88)

(31) Priority Application Number: 131,390

(32) Priority Date: 10 December 1987 (10.12.87)

(33) Priority Country: US

(71) Applicant: LA JOLLA CANCER RESEARCH FOUNDATION [US/US]; 10901 North Torrey Pines Road, La Jolla, CA 92037 (US).

(72) Inventors: PIERSCHBACHER, Michael, D. ; 1111 Alexandria Drive, San Diego, CA 92107 (US). RUOSLAHTI, Erkki, I. ; P.O. Box 1054, Rancho Santa Fe, CA 92067 (US).

(74) Agents: CAMPBELL, Cathryn et al.; Pretty, Schroeder, Brueggemann & Clark, 444 South Flower Street, Suite 2000, Los Angeles, CA 90071 (US).

(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).

Published*With international search report.**Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.*

(54) Title: CONFORMATIONALLY STABILIZED CELL ADHESION PEPTIDES

(57) Abstract

Novel synthetic Arg-Gly-Asp containing peptides which have high affinity and specificity for their receptors by virtue of restrictions on their stereochemical conformation. Such restrictions can be provided by cyclization, by inclusion into a constraining conformational structure such as a helix, by providing an additional chemical structure such as an amide or an enantiomer of a naturally occurring amino acid, or by other methods. In particular, there are provided cyclic peptides having increased affinity and selectivity for the certain receptors over that of linear, Arg-Gly-Asp-containing synthetic peptides.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT Austria
AU Australia
BB Barbados
BE Belgium
BG Bulgaria
BJ Benin
BR Brazil
CF Central African Republic
CG Congo
CH Switzerland
CM Cameroon
DE Germany, Federal Republic of
DK Denmark
FI Finland

FR France
GA Gabon
GB United Kingdom
HU Hungary
IT Italy
JP Japan
KP Democratic People's Republic
of Korea
KR Republic of Korea
LI Liechtenstein
LK Sri Lanka
LU Luxembourg
MC Monaco
MG Madagascar

ML Mali
MR Mauritania
MW Malawi
NL Netherlands
NO Norway
RO Romania
SD Sudan
SE Sweden
SN Senegal
SU Soviet Union
TD Chad
TG Togo
US United States of America

-1-

CONFORMATIONALLY STABILIZED CELL ADHESION PEPTIDES
Field of the Invention

This invention relates generally to peptides and more specifically to peptides which are involved in cell adhesion systems.

5 Background of the Invention

The adhesion of cells to other cells or to extracellular materials is important to the orderly development and functioning of cells in an organism. Cell adhesion is mediated by certain adhesive legands and corresponding
10 receptors. Among such adhesive ligands are the glyco- proteins fibronectin, vitronectin and collagen. All three contain the tripeptide sequence arginine-glycine-aspartic acid (Arg-Gly-Asp or R-G-D) which appears to function as the primary recognition site for receptors on the surface
15 of cells binding to these molecules. When presented to cells as a peptide-covered surface, synthetic peptides containing the Arg-Gly-Asp sequence promote cell attachment in a manner similar to that of natural fibronectin or vitronectin. In solution, such peptides can inhibit cell
20 attachment to a surface coated with fibronectin, vitronectin, collagen, the peptides themselves or some other adhesive protein having an Arg-Gly-Asp cell attachment site.

Several receptors have now been identified which
25 recognize the Arg-Gly-Asp sequence of their respective ligands. While some of these receptors have affinity for only their specific ligand, others will interact to varying degrees with two or more ligands containing the tripeptide. It therefore appears that while the Arg-Gly-Asp sequence is
30 sufficient for receptor recognition and binding, the remaining amino acid sequence of the peptide may nonetheless be important to the specificity of the ligand-receptor interaction. The precise manner in which the remaining

-2-

sequence affects binding has, thus far, remained elusive.

One view that has been held by some investigators in the field is that the specificity of a given adhesive protein for its receptor may depend on the presence in the adhesive protein of one or more receptor binding sites other than their Arg-Gly-Asp site. According to this view, the Arg-Gly-Asp site would be a shared binding site and the additional site or sites would be responsible for the specificity (See, for example, Yamada et al., (1987) Biochem. and Biophys. Res. Comm. 144:35; Wright et al., (1987) PNAS 84:1965). An alternative possibility is that the Arg-Gly-Asp sequence provides essentially all of the information for the receptor binding and that it is the conformation of this sequence that gives an adhesion protein its receptor specificity. The binding sites of various peptide hormones are known to be small (Rose et al., (1985) Adv. in Chemistry 37:1) but these peptides generally comprise only about 10 amino acids in total. In contrast, each of the two fibronectin polypeptide chains comprises over 2000 amino acids. The idea that the conformation of a single amino acid triplet could be important to the function of a number of different proteins carrying it is novel.

Synthetic peptides containing the Arg-Gly-Asp sequence are useful in that they can both promote and inhibit cell attachment. There are at least ten different adhesion receptors that are known or suspected to recognize an Arg-Gly-Asp sequence in one or more of the various adhesive proteins. Peptides that reproduce with reasonable accuracy the function of such adhesive proteins or specifically inhibit that function offer a tremendous potential for the manipulation of important physiological events such as thrombosis, metastasis, inflammation, wound healing, and rejection of prosthetic implants.

There thus exists a need for peptides having an amino acid structure that provides the optimum specificity for

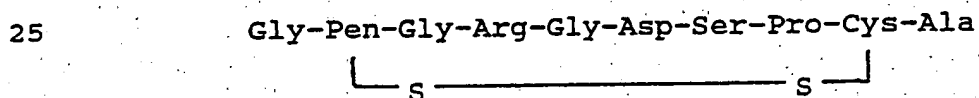
-3-

the receptor of interest. The present invention satisfies this need and provides related advantages as well.

Summary of the Invention

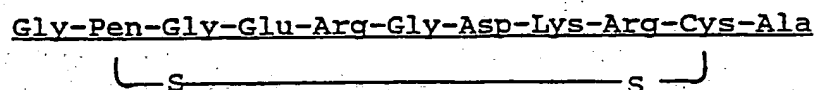
The present invention involves novel synthetic peptides 5 which have high affinity and specificity for their receptors by virtue of restrictions on their stereochemical conformation. Such restrictions, or stabilizations, can be provided, for example, by cyclization, by inclusion into a constraining conformational structure such as a helix, by 10 providing an additional chemical structure such as an amide or an enantiomer of a naturally occurring amino acids, or by other methods. Such peptides can have a range of numbers of residues, but preferably between 3 and 100, more preferably 7 to 30. In particular, there is provided a 15 cyclic peptide having increased affinity and selectivity for the vitronectin receptor over that of linear, Arg-Gly-Asp-containing synthetic peptides. The affinity and selectivity of the cyclic peptide approaches that of natural vitronectin.

20 In one aspect, the invention comprises a peptide having about 10 amino acids in which a bridge is formed between amino acids surrounding the Arg-Gly-Asp sequence. A suitable structure is

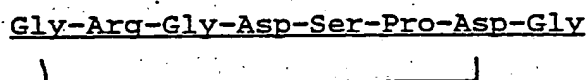


The peptide may have about 1 to 4 amino acids between the Arg-Gly-Asp sequence and those residues forming the bridge. The bridging residues may be, for example, penicillamine 30 and cysteine, or other amino acids capable of forming a bridge, such as a disulfide bridge.

Another suitable structure is ⁻⁴⁻

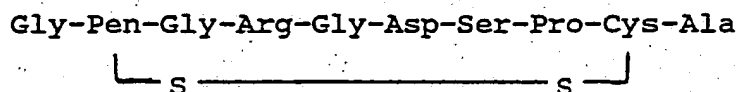


- 5 Alternatively, the peptide may be cyclized through a peptide bond between amino acid residues. An appropriate structure is



- 10 The present invention provides evidence that, rather than the specificity of adhesion ligands residing in distinct binding sites outside the Arg-Gly-Asp site, such specificity results from the conformational structure imposed on the Arg-Gly-Asp sequence by the structure of the
 15 remaining peptide. At the practical level, the invention demonstrates that a conformationally restricted Arg-Gly-Asp-containing peptide can have different receptor specificity and higher binding affinity than its unrestricted counterpart. This demonstration permits the
 20 synthesis of other Arg-Gly-Asp conformations with variant receptor specificities and affinities.

The stabilized peptides of the present invention have various applications relating to their cell-adhesion properties. In one embodiment, for example, where the
 25 peptide structure is



- the peptide has increased affinity for the vitronectin receptor and decreased affinity for the fibronectin
 30 receptor over that of linear synthetic Arg-Gly-Asp-containing peptides and may therefore be effectively used to inhibit the binding of vitronectin receptor-containing cells to a substrate for cell culture. Alternatively, the stabilized peptide may be usefully employed to promote

-5-

adhesion of cells expressing the vitronectin receptor by, for example, coating cell culture substrates. Additionally, the stabilized peptide may be used to coat materials for in vitro implantation, such as prothesis, where cell attachment is desired.

Brief Description of the Drawings

The present invention will be described in connection with the accompanying drawings in which:

FIGURE 1 is a graph of HPLC analysis of a crude preparation of cyclic peptide (a); and purified cyclic peptide (b).

FIGURE 2 is a graph illustrating adhesion of normal rat kidney cells to fibronectin and vitronectin in the presence of a cyclic and a linear peptide having the same amino acid sequence.

FIGURE 3 shows the sequence of the peptide of Example III and the recovery of its degradation products recovered by automated Edman degradation.

FIGURE 4 records the change in optical rotation caused by a solution of the peptide of Fig. 3, as a function of increasing temperature, indicating its restricted conformation at the lower temperature.

FIGURE 5 is an axial projection of the peptide of Example IV. Those residues above the dotted line are lipophilic while those below the line are hydrophilic.

FIGURE 6 is a schematic representation of the cyclic peptide synthesized in Example VI.

Detailed Description of the Invention

As used herein, the term "conformationally stabilized"

-6-

or "conformationally constrained" refers to limitations imposed on the number of possible stereochemical structures which a peptide may assume. In the context of the present invention, such limitations are imposed on the conformation of the Arg-Gly-Asp binding site and result from the presence of chemical structures surrounding the binding site which limit the possible structural conformations of the binding site to less than those assumable by the tripeptide sequence alone. Such conformationally stabilized Arg-Gly-Asp-containing peptides still exhibit binding activity with at least one receptor. "Surrounding chemical structures" may refer either to amino acids or other chemical moieties, and may refer both to those moieties lying immediately adjacent the binding site as well as those separated from the binding site itself by the presence of intervening amino acids. The term "amino acids surrounding the Arg-Gly-Asp sequence" refers both to residues adjacent to the Arg-Gly-Asp sequence as well as to residues separated from the Arg-Gly-Asp sequence by intervening residues.

As used herein, references to "Arg-Gly-Asp containing peptides" are intended to refer to peptides having one or more Arg-Gly-Asp containing binding sites which may function as binding sites for a receptor of the "Arg-Gly-Asp family of receptors", i.e., those recognizing and binding to the Arg-Gly-Asp sequence. While the Arg-Gly-Asp sequence has been found to necessarily be invariant in order to retain the binding activity, the composition of the remaining peptide as well as any other chemical moiety present in conjunction with the peptide may vary without necessarily affecting the activity of the binding site. Where specific chemical structures or sequences beyond the Arg-Gly-Asp sequence are presented, it is intended that various modifications which do not destroy the function of the binding site are to be encompassed without departing from the definition of the peptide.

As used herein, the term "bridge" refers to a chemical

-7-

bond between two amino acids in a peptide other than the amide bond by which the backbone of the peptide is formed.

As used herein, the term "peptide bond" or "peptide linkage" refers to an amide linkage between a carboxyl group of one amino acid and the α -amino group of another amino acid.

The abbreviations for amino acids are used herein are given in the following table:

	Ala	Alanine
10	α -ABA	α -Amino isobutyric acid
	Arg	Arginine
	Asp	Aspartic acid
	Cys	Cysteine
	Glu	Glutamic acid
15	Gly	Glycine
	Leu	Leucine
	Lys	Lysine
	Pen	Penicillamine
	Pro	Proline
20	Ser	Serine
	SuccAla	Succinyl-alanine

The present invention provides novel synthetic peptides which incorporate the sequence Arg-Gly-Asp in a conformationally stabilized form. In one embodiment, the peptide comprises the sequence X-R₁-R₂-Arg-Gly-Asp-R₃-R₄-Y in which R₁ and R₄ are amino acids forming or capable of forming a bridge, such as a disulfide bridge, or a peptide linkage, R₂ and R₃ are each a sequence of 0 to 5 amino acids, X is one or more amino acids or H and Y is one or more amino acids or OH or NH₂ and together X and Y preferably total about zero to 100 amino acids, although longer sequences may also be useful. In a preferred embodiment, R₁ is penicillamine, R₄ is cysteine, R₂ is glycine and R₃ is Ser-Pro. Additional amino acids can be present at the NH₂ and COOH termini, as represented by X and Y, respectively.

-8-

Such peptides may be synthesized by any suitable method, including well-known methods of chemical synthesis. Preferably, the linear sequence is synthesized using commercially available automated peptide synthesizers. The material so synthesized can be precipitated and further purified, for example by high performance liquid chromatography (HPLC). Although a purity of greater than 95 percent for the synthesized peptide is preferred, lower purity may be acceptable.

- 10 To obtain one of the stabilized peptides of the present invention which has high specificity and affinity for particular cell surface receptors related to the cell adhesion system, the synthesized peptide is cyclized using methods well known in the art. For example, where the
15 residues contain a sulfhydryls, a disulfide bridge may be formed by oxidizing a dilute aqueous solution of the peptide with $K_3[Fe(CN)_6]$. Other means of cyclizing which are known in the art may also be utilized.

The stabilized cyclized peptides of the present
20 invention can also be prepared by forming a peptide bond between non-adjacent amino acid residues. A procedure for forming such peptide bond is provided in Schiller et al., Int. J. Peptide Protein Res. 25:171(1985), which is incorporated herein by reference. Briefly, cyclic peptides
25 can be synthesized on the Merrifield resin by assembling the peptide chain using N^α -Fmoc-amino acids and Boc and tertiary-butyl protein.

The stabilized peptide can also be prepared by designing or treating it so as to form a helix such as an alpha (α)
30 helix or a triple helix, according to methods well-known in the art.

The stabilized peptides described herein can be used as analogues to adhesion proteins including, for example, vitronectin. Because of the increased affinity for the

-9-

vitronectin receptor of the cyclic peptide of the preferred sequence over that of the analogous linear sequence, the cyclic peptide may be used to inhibit binding of vitronectin without affecting the function of other
5 receptors such as the fibronectin receptor. Alternatively, the cyclic peptide can be used to promote adhesion of cells expressing the vitronectin receptor, as by coating cell culture substrates.

Certain specific structures containing an Arg-Gly-Asp
10 sequence have been found to confer particular specificity or other attribute on the binding site. Among such structures are the sequences -Arg-Gly-Asp-NH₂, -D-Arg-Gly-Asp- and -Arg-Gly-Asp-D-Ser-. The conformationally stabilized latter sequence exhibits useful affinity for the
15 fibronectin receptor but not the vitronectin receptor. The presence of the enantiomeric forms of the naturally occurring amino acids has been shown to confer resistance to degradation enzymes, such as trypsin, in the case of D-Arg. Other useful peptides include the sequences Phe-Arg-
20 Gly-Asp-Ser-Pro, Gly-Arg-Gly-Asp-Ser-Phe, and Phe-Arg-Gly-Asp-Ser-Phe.

The peptides of the present invention may be utilized industrially for in vivo uses such as coating of medical devices, including prostheses or implants, for example
25 vascular implants, so as to facilitate the attachment of cells thereto. In addition, the peptides have in vitro uses in coating of substrates, such as cell culture substrates, to promote cell adhesion.

Other features and advantages of the present invention
30 will become apparent from the following more detailed Examples which illustrate, by way of example, the principles of the invention.

-10-

EXAMPLE I

Peptide Synthesis

The peptide Gly-Pen-Gly-Arg-Gly-Asp-Ser-Pro-Cys-Ala was synthesized using an automated peptide synthesizer (Model 5 430A; Applied Biosystems, Foster City, California) according to the directions provided by the manufacturer. After cleavage from the resin with hydrogen fluoride, the peptides were washed in cold ethyl ether and precipitated from solution in trifluoroacetate with ice cold ether. The 10 peptides were then redissolved in distilled water and lyophilized. The peptides were further purified by HPLC using a Waters Bondapak™ C₁₈ (3 x 30 cm; 10 μm packing, Waters Assoc., Milford, MA).

EXAMPLE II

15

Cyclization of the Peptide

611 mg of the peptide synthesized as described in Example I were dissolved in 4 liters of water that had been previously boiled and allowed to cool. Immediately prior to addition of the peptide, nitrogen was bubbled through 20 the water for 45 minutes. After the peptide was dissolved, a solution of 0.1 μg/ml of potassium ferrous cyanide K₃[Fe(CN)₆] in water was added dropwise to the stirred peptide solution until the yellow color persisted for 5 minutes (approximately 5 ml). The pH of the solution was 25 held at 7.0 throughout this procedure by addition of NH₄OH. The solution was allowed to stand for 20 hours under low vacuum and then lyophilized. Excess K₃[Fe(CN)₆] was removed by passing the cyclized material over a Sephadex G-15 column (1.8 x 120 cm). The peptide was purified by 30 reverse phase HPLC using a Waters Bondapak™ C₁₈ column (3 x 30 cm; 10 μm packing) (Waters Assoc., Milford, MA). The peptide was loaded on the column in buffer A (20 mM ammonium acetate at pH 7.5) and eluted with a gradient of buffer B consisting of 60% acetonitrile and 40% buffer A.

Figure 1 depicts the result of ⁻¹¹⁻ purification of the cyclized peptide by reverse phase HPLC. Trace a represents purification of the peptide on the C₁₈ column. Fractions 1, 2 and 3 were pooled for testing of their ability to inhibit attachment of cells to fibronectin or vitronectin. Fraction 2, containing cyclic peptide, was rechromatographed by the same procedure on a C₁₈ column and yielded a single peak (trace b).

The major peak obtained from the C₁₈ column (fraction 2 in Figure 1) constituted 90% of recovered peptide and was deduced to be a monomeric cyclic peptide because it was retained on the column for the length of time predicted for that sequence and because the uncyclized material and multimeric forms were well separated from the main peak.

15

EXAMPLE III

Formation of a Triple Helical Structure

The cell attachment promoting Arg-Gly-Asp sequence of the linear peptide generated as in Example I was conformationally restrained by allowing the peptide to assume a helical structure, according to methods of Dedhar et al, (1987) J. Cell Biol. 104:585, which is incorporated hereby reference. Briefly, a 33-amino acid peptide having the sequence (Pro-Hyp-Gly)₄-Ala-Pro-Gly-Leu-Arg-Gly-Asp-Thr-Gly-(Pro-Hyp-Gly)₄ was synthesized using an automated peptide synthesizer (Model 430A; Applied Biosystems, Foster City, CA). The sequence was confirmed by automated Edman degradation using a gas phase sequencer (Model 470A; Applied Biosystems, Foster City, CA) as shown in Figure 3. The linear peptide was allowed to dissolve overnight in the smallest possible volume of 0.05% acetic acid at 4°C. Polarimetrical analysis was used to confirm that it had assumed a helical structure according to the method of Rhodes et al., (1978) Biochemistry, 17:3442, which is incorporated herein by reference. The peptide existed as a triple helix at low temperatures as judged by the high

-12-

degree of negative optical rotation which underwent a sharp change as the temperature was increased, producing a "melting" curve having an inflection point (second derivative equal to 0; T_m) at 30°C., as shown in Figure 4.

5

EXAMPLE IV

Formation of an Alpha Helical Structure

An Arg-Gly-Asp containing peptide was designed so as to have an amino acid sequence such that it would under appropriate conditions assume an alpha helical configuration. As demonstrated by Carbone et al., (1987) J. Immunology 138:1838, which is incorporated herein by reference, peptides comprising alternating α -aminoisobutyric acid (α -ABA) and alanine (Ala) residues adopt helical conformations in structure-forming organic solvents. Moreover, the presence of a negatively charged group at the amino terminus and a positively charged group at the carboxy terminus creates a dipole which acts to stabilize such a helix (Shoemaker et al., (1987), Nature 326:563, which is incorporated herein by reference). Further, the sequence of amino acids were chosen so as to create an amphiphilic secondary structure in which hydrophilic residues lie on one side, lipophilic residues on the other side of the helix (Kaiser et al., (1984) Science, 223:249, which is incorporated herein by reference).

25 The following linear sequence was produced by an automated peptide sequencer (Model 430A; Applied Biosystems, Foster City, CA) was: SuccAla-Leu-Glu-Glu- α ABA-Ala-Lys-Arg-Gly-Asp-Ser-Leu- α ABA-Gly-Lys- α ABA-Ala-Lys.

The peptide was synthesized and purified according to the methods of Example 1. The alpha helical conformation of this peptide is depicted schematically in Fig. 5.

-13-
EXAMPLE V

Cell Adhesion Assay

Fibronectin and vitronectin were purified from human plasma according to the methods of Ruoslahti et al., (1982) 5 Meth. Enz. 82:803; and Hayman et al., (1983) PNAS 80:4003, respectively, both of which are incorporated herein by reference. The adhesion of normal rat kidney cells to fibronectin and vitronectin was assayed as described in Ruoslahti et al., Meth. Enz. supra. Briefly, 0.1 ml 10 solution of fibronectin or vitronectin at a concentration of 12 μ g/ml in 0.1M PBS was placed in each well of 96-well plastic microtiter plates and left for 2 hours at room temperature. The unattached protein was removed by washing three times with PBS.

15 One day before the assay, a confluent culture of normal rat kidney cells was split 1:2 using standard tissue culture trypsin. The cells were washed three times with PBS, pH 7.2. 10 ml of 2 x crystallized trypsin (Sigma, Type III), 0.1 mg/ml in PBS, was added, and the cells 20 incubated at 37°C until they detached. The detached cells were then collected by centrifugation and washed three times with a solution of 0.5 mg/ml of soybean trypsin inhibitor in PBS to ensure neutralization of the trypsin. Before the last centrifugation, a sample was taken and cell 25 numbers and viability by trypan blue exclusion are determined. The cells were suspended in minimal essential medium (MEM) at a concentration of 10^6 cells/ml and dispersed by pipetting until a single-cell suspension was obtained.

30 To ensure even dispersal of the cells in the microtiter well, 0.1 ml of MEM was added to each well, followed by the addition of 0.1 ml of the cell suspension. The plate was incubated for 1 hour at 37°C.

The unattached cells were removed simply by removing the

-14-

medium and washing the wells. The plate was flooded with PBS, and the washing solution was removed. The cells were then fixed with 3% paraformaldehyde in PBS and stained with 1% toluidine blue, paraformaldehyde in PBS, and attached cells counted. Their number was determined using a cell counter that is capable of counting cells attached to a surface (Dynatech Laboratories, Alexandria, VA).

The ability of the cyclized peptide of the invention to inhibit adhesion of cells to the substrates was assayed by adding stock solutions of the peptide dissolved in MEM to give final concentrations in the wells of 0 to 10.0 mM. Prior to addition to the assay, the stock solutions were neutralized with sodium bicarbonate. Rat kidney cells were added and the wells incubated and assayed as described.

In order to more fully determine the ability of the cyclized peptide to inhibit the attachment of normal rat kidney cells to the fibronectin and vitronectin substrates, the binding of a non-cyclized, or linear, peptide having the sequence Gly-Pen-Gly-Arg-Gly-Asp-Ser-Pro-Cys was assayed. As shown in Figure 2, the cyclic peptide of the invention inhibited attachment to vitronectin at a 10-fold lower molar concentration than the linear peptide, but it was ineffective at inhibiting attachment to fibronectin. The linear peptide, in contrast, inhibited attachment of cells to both substrates at relatively similar concentrations.

While not wishing to be bound by this explanation, the cyclic peptide of the invention appears to possess a different affinity for the binding site of fibronectin and vitronectin receptors. This is consistent with the fact that the rat kidney cells used in the assay are known to possess separate receptors for fibronectin and vitronectin.

EXAMPLE VI

Cyclization Through Peptide Bond

The peptide cyclo-(1-7)Gly-Arg-Gly-Asp-Ser-Pro-Asp-Gly was synthesized using a modification of the method of 5 Schiller et al., Int. J. Peptide Protein Res. 25:171 (1985), which is incorporated herein by reference.

The synthesis was done using PAM resin-TFA; (Applied Biosystems, Inc., Foster City, CA) with t-Boc Gly-attached to the resin with a substitution factor of 0.80 mmol/g 10 (see Figure 6). All amino acids were obtained from Bachem Bioscience, Inc., Philadelphia, PA. 1.25 g (1.0 mmol) of resin was shaken with 20 ml dichloromethane (DCM) for 15 minutes to induce swelling and DCM removed with N₂ pressure the procedure repeated. The t-Boc protecting group was 15 cleaved with 20 ml 20% TFA; (Applied Biosystems, Inc., Foster City, CA) in DCM (Pfizer, Groton, CN) for 15 minutes. This reaction was repeated, this time shaking for 30 minutes. Between the reactions, the resin was washed once with 20 ml DCM for 2 minutes. After the second 20 reaction, the resin was washed six times with 20 ml DCM for 2 minutes each, followed by neutralization with 20 ml 10% triethylamine (TEA) in DCM two times for 3 minutes each. The resin was washed six times with 20 ml DCM for 2 minutes each and was then ready for the next reaction.

25 In a 50 ml round bottom flask equipped with stirring bar, Fmoc-Asp-t-butyl ester ((OtBu)OH) (1.03 g, 2.5 mol) was dissolved in 15 ml DCM followed by addition of 2.5 ml (1M) DIC (Aldrich Chem. Co., Milwaukee, WI) in DCM. The mixture diisopropyl carbodiimide was stirred for 5 minutes 30 and followed by addition of 1.25 ml 2.0 M hydroxybenzotriazole (HOBT; Aldrich) in DMF (Pfizer) at 0°C and stirred for 10-15 minutes more. This mixture was added to the resin and shaken 1 to 2 hours. This reaction was repeated once (double coupled) and the resin was washed 2 35 times with 20 ml DCM between couplings. After the second

-16-

coupling was completed, the resin was washed three times with 20 ml DCM.

The cleavage of the Fmoc group was done with 20 ml 20% piperidine (Aldrich) in DCM (freshly made) and shaken for 15 minutes. This reaction was repeated for another 30 minutes. The resin was then washed once with DMF and five times with DCM for 2.0 minutes each. Then the resin was ready for the next coupling.

In a 50 ml round bottom flask equipped with stirring bar, Fmoc-Pro-OH (0.84 g, 2.5 mmol) was dissolved in 15 ml DCM followed by addition of 2.5 ml (1 M) DIC in DCM. The mixture was stirred for 5.0 minutes, followed by the addition of 1.25 ml (2M) HOBT in DMF at 0°C and stirred for 10 to 15 minutes more. This mixture was added to the resin and shaken 1 to 2 hours. This reaction was repeated once (double coupled) and the resin was washed 2 times with 20 ml DCM between couplings. After the second coupling was completed, the resin was washed three times with 20 ml DCM. The Fmoc was cleaved using the same procedure as above and the material was then ready for the next coupling.

In a 50 ml round bottom flask equipped with stirring bar, Fmoc-Asp-benzyl ester ((OBzl)-OH) (1.11 g, 2.5 mmol) was dissolved in 15 ml DCM followed by addition of 2.5 ml (1 M) DIC in DCM. The mixture was stirred for 5.0 minutes, followed by addition of 1.25 ml 2.0 M HOBT in DMF at 0°C and stirred for 10 to 15 minutes. This mixture was added to the resin and shaken 1 to 2 hours. This reaction was repeated once (double coupled) and the resin was washed 2 times with 20 ml DCM between couplings. After the second coupling was completed the resin was washed three times with 20 ml DCM. The Fmoc was cleaved using the same procedure as above and the material was then ready for the next coupling.

In a 50 ml round bottom flask equipped with stirring bar, Fmoc-Gly-OH (0.74 g, 2.5 mmol) was dissolved in 15 ml

-17-

DMF followed by addition of 2.5 ml (1 M) DIC in DCM. The mixture was stirred for 5.0 minutes, followed by addition of 1.25 ml 2.0 M HOBT in DMF at 0°C and stirred for 10 to 15 minutes more. This mixture was added to the resin and 5 shaken 1 to 2 hours. This reaction was done twice (double coupled) and the resin was washed 2 times with 20 ml DCM in between coupling. After the second coupling was completed the resin was washed three times with 20 ml DCM. The Fmoc was cleaved using the same procedure as above and the 10 material was then ready for the next coupling.

In a 50 ml round bottom flask equipped with stirring bar, Fmoc-Arg Tosilate ((Tos)OH) (1.37 g, 2.5 mmol) was dissolved in 15 ml DCM followed by addition of 2.5 ml (1M) DIC in DCM. The mixture was stirred for 5.0 minutes and 15 followed by addition of 1.25 ml 2.0 M HOBT in DMF at 0°C and stirred for 10 to 15 minutes more. This mixture was added in to the resin and shaken 1 to 2 hours. This reaction was repeated once (double coupled) and the resin was washed 2 times with 20 ml DCM in between coupling. 20 After the second coupling was done the resin was washed three times with 20 ml DCM. The Fmoc deprotection was performed using the same as the procedure above and the material was then ready for the next coupling.

In a 50 ml round bottom flask equipped with stirring 25 bar, t-Boc-Gly-OH (0.44 g, 2.5 mmol) was dissolved in 15 ml DCM followed by addition of 2.5 ml (1M) DIC in DCM. The mixture was stirred for 5.0 minutes and followed by addition of 1.25 ml (2M) HOBT in DMF at 0°C and stirred for 10 to 15 minutes more. This mixture was added in to the 30 resin and shaken 1 to 2 hours. This reaction was repeated (double coupled) and the resin was washed 2 times with 20 ml DCM in between coupling for 2 minutes each. After the second coupling was done the resin was washed three times with 20 ml DCM for 2 minutes each.

35 The deprotection of the t-Boc Group of Gly 1 and the t-Butyl ester side chain-of-Asp 7 was performed at the same

-18-

time by adding 20 ml 20% TFA in DCM for 15 minutes. This reaction was repeated once again for 30 minutes. Between reactions the resin was washed once with DCM for 2 minutes. After the second reaction, the resin was washed six times with 20 ml DCM for 2 minutes each, followed by neutralization with 20 ml 10% TEA in DCM two times for 3 minutes each. The resin was washed six times with 20 ml DCM for 2.0 minutes each.

The N-terminus of Gly 1 was cyclized to the side chain 10 carboxyl group of Asp 7. The cyclization was accomplished by adding 10 ml of 1M DIC solution and 5 ml 2M HOBT in DMF and shaken for 4 days. Each day, old reagent was drained and the resin was washed with 20 ml DMF once and with DCM three times for 2 minutes each. Fresh reagent (DIC and 15 HOBT) was then added.

The resin was cleaved with hydrogen fluoride using procedures well known in the art. After cleaving, the peptide-containing resin was washed with cold ethyl ether then dissolved with 10% acetic acid to remove the 20 peptide from the resin. The filtrate was frozen and lyophilized. The crude product recovered was 508 mg. This crude product was subjected to purification using HPLC through C₁₈ (1 X 20 cm; 10- μ m packing) column. The peptide was loaded on the column in buffer A (10 mM ammonium 25 acetate at pH 6.0) and eluted with a gradient of buffer B consisting of 60% acetonitrile and 40% buffer A. Yield after purification was 210 mg.

This compound was confirmed to have the desired cyclic peptide structure by proton NMR in DMSO-d₆ as the solvent. 30 The 1D and 2D (COSY, COSY-Relay, and NOESY) spectra of this compound were obtained by using a 360 MHz FT-NMR equipped with Nicolet software at the University of California, San Diego NMR facility. (See Wuthrich, K., NMR of Proteins and Nucleic Acids, John Wiley & Son, NY (1986), which is 35 incorporated herein by reference).

-19-

Although the invention has been described with reference to the presently-preferred embodiment, it should be understood that various modifications can be made by those skilled in the art without departing from the invention.

5 Accordingly, the invention is limited only by the following claims.

WE CLAIM:

-20-

1. A method of synthesizing an Arg-Gly-Asp-containing ligand for a member of the Arg-Gly-Asp-receptor family, comprising combining an Arg-Gly-Asp sequence with an additional chemical structure so as to conformationally restrict the stereochemical structure of said Arg-Gly-Asp sequence.
2. The method of claim 1 wherein said conformational restriction is effected by forming a cyclic peptide through a bridge between amino acids surrounding the Arg-Gly-Asp sequence.
3. The method of claim 2 wherein said bridge is a disulfide bridge.
4. The method of claim 2 wherein said bridge is a peptide bond.
5. The method of claim 1 wherein said conformational restriction is effected by inserting the Arg-Gly-Asp containing peptide into a helical structure.
6. The method of claim 5 wherein said helical structure is a triple helix.
7. The method of claim 5 wherein said helical structure is an alpha helix.
8. The method of claim 1 wherein said conformational restriction is effected by providing the Arg residue in the D form.
9. The method of claim 1 wherein said conformational restriction is effected by combining an Arg-Gly-Asp-containing peptide with an additional chemical moiety.

-21-

10. The method of claim 9 wherein said additional chemical moiety is a D-serine at the carboxy terminus of said Arg-Gly-Asp sequence.

11. The method of claim 9 wherein said additional chemical moiety is a phenylalanine at the amino terminus of the Arg-Gly-Asp sequence.

12. The method of claim 9 wherein said additional chemical moiety is a peptide.

13. A product made by the process of claim 1.

14. A method of increasing the specificity of an Arg-Gly-Asp-containing ligand for a member of the Arg-Gly-Asp receptor family comprising combining an Arg-Gly-Asp sequence with an additional chemical structure so as to 5 conformationally restrict the stereochemical structure of said Arg-Gly-Asp sequence in such a way that the affinity of the Arg-Gly-Asp binding site sequence for a particular receptor is enhanced.

15. The method of claim 14 wherein said conformational restriction is effected by forming a cyclic peptide through a bridge between amino acids surrounding the Arg-Gly-Asp sequence.

16. The method of claim 15 wherein said bridge is a disulfide bridge.

17. The method of claim 15 wherein said bridge is a peptide bond.

18. The method of claim 14 wherein said conformational restriction is effected by inserting the Arg-Gly-Asp containing peptide into a helical structure.

19. The method of claim 18 wherein said helical structure is an alpha helix.

-22-

20. The method of claim 18 wherein said helical structure is an alpha helix.

21. The method of claim 14 wherein said conformational restriction is effected by providing the Arg residue in the D form.

22. The method of claim 14 wherein said conformational restriction is effected by combining the Arg-Gly-Asp containing peptide with an additional chemical moiety.

23. The method of claim 22 wherein said additional chemical moiety is a D-serine at the carboxy terminus of said Arg-Gly-Asp sequence.

24. The method of claim 22 wherein said additional chemical moiety is a phenylalanine residue at the amino terminus of the Arg-Gly-Asp sequence.

25. A composition of matter comprising an Arg-Gly-Asp-containing peptide and an additional chemical structure combined so as to restrict the stereochemical structure of the Arg-Gly-Asp sequence.

26. A peptide containing the sequence -R₁-R₂-Arg-Gly-Asp-R₃-R₄- in which R₂ comprises about 0 to 5 amino acids and R₃ comprises about 0 to 5 amino acids and wherein R₁ and R₄ are amino acids connected by a bridge.

27. A stabilized peptide including the cell attachment promoting sequence Arg-Gly-Asp in which the Arg-Gly-Asp sequence is conformationally restricted.

28. The stabilized peptide of claim 27 wherein the cell attachment promoting Arg-Gly-Asp sequence is conformationally restricted by forming a cyclic peptide through a bridge between amino acids surrounding the Arg-Gly-Asp sequence.

-23-

29. The stabilized peptide of claim 28 wherein said bridge is a disulfide bridge.

30. The stabilized peptide of claim 28 wherein said bridge is a peptide bond.

31. The stabilized peptide of claim 27 wherein said conformational restriction is effected by inserting the Arg-Gly-Asp-containing peptide into a helical structure.

32. The stabilized peptide of claim 31 wherein said helical structure is a triple helix.

33. The stabilized peptide of claim 31 wherein said helical structure is an alpha helix.

34. The stabilized peptide of claim 27 wherein said conformational restriction is effected by combining the Arg-Gly-Asp containing peptide with an additional chemical moiety.

35. The stabilized peptide of claim 34 wherein said additional chemical moiety is a D-serine at the carboxy terminus of the Arg-Gly-Asp sequence.

36. The stabilized peptide of claim 34 wherein said additional chemical moiety is a phenylalanine residue at the amino terminus of the Arg-Gly-Asp sequence.

37. The stabilized peptide of claim 34 wherein said additional chemical moiety is a peptide.

38. A method of inhibiting attachment of cells in culture to a substrate comprising the steps of:

a. providing a stabilized peptide of claim 27 in solution; and

5 b. contacting said cells in culture with said stabilized peptide in solution.

-24-

39. A method of promoting cell attachment to a substrate comprising the steps of:

- a. immobilizing the peptide of claim 27 on a substrate to form a stabilized peptide-associated substrate; and
- b. exposing free cells in culture to said stabilized peptide-associated substrate.

40. A cyclized peptide comprising the sequence
Gly-Pen-Gly-Arg-Gly-Asp-Ser-Pro-Cys-Ala



41. A peptide having cell binding activity containing one or more of the following sequences:

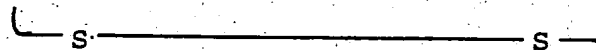
Arg-Gly-Asp-NH₂;

Phe-Arg-Gly-Asp-Ser-Pro;

5 Gly-Arg-Gly-Asp-Ser-Phe; or

Phe-Arg-Gly-Asp-Ser-Phe.

42. A cyclized peptide comprising the sequence
Gly-Pen-Gly-Glu-Arg-Gly-Asp-Lys-Arg-Cys-Ala



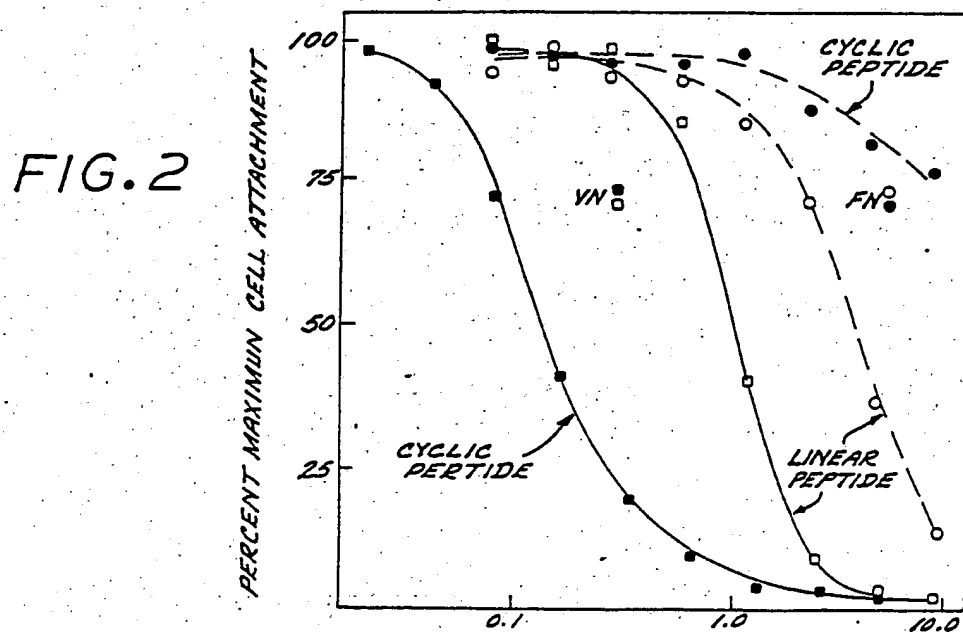
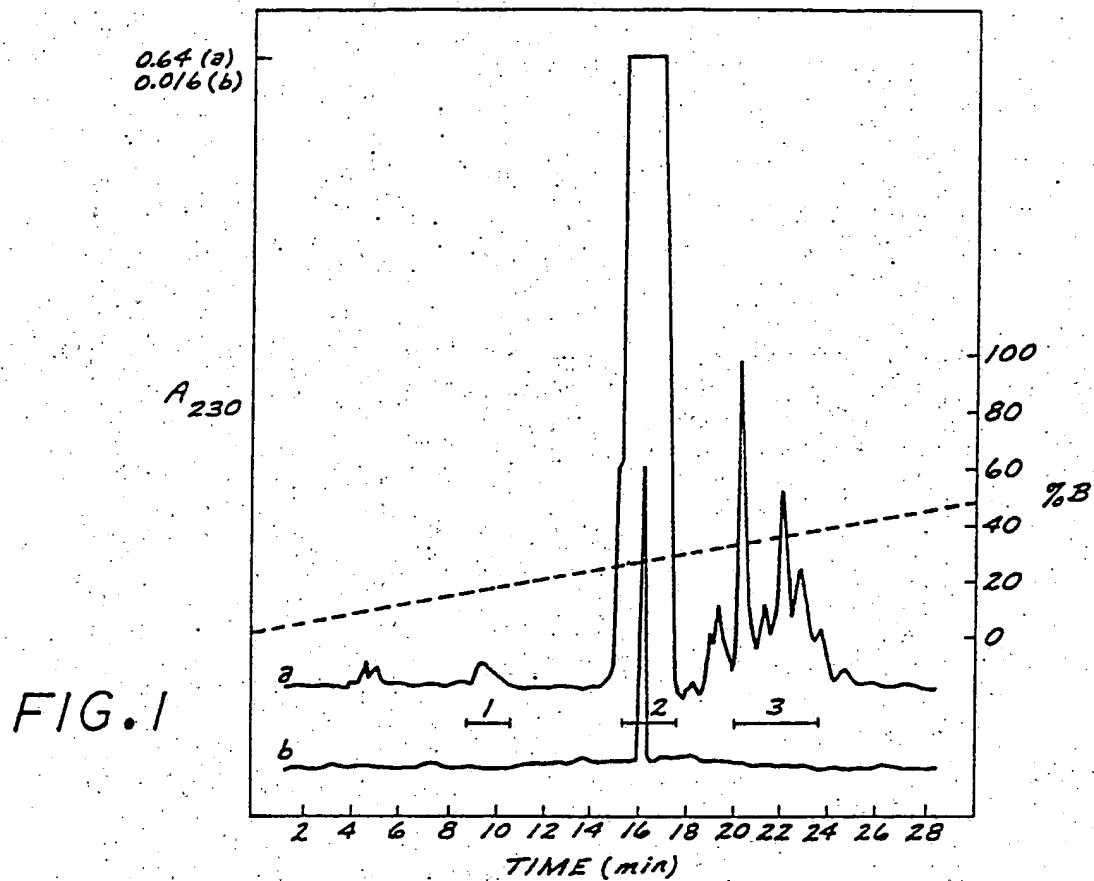
43. A cyclized peptide comprising the sequence
Gly-Arg-Gly-Asp-Ser-Pro-Asp-Gly



44. A cyclized peptide comprising the sequence
Gly-Pen-Gly-His-Arg-Gly-Asp-Leu-Arg-Cys-Ala



2/4



2/4

FIG. 3

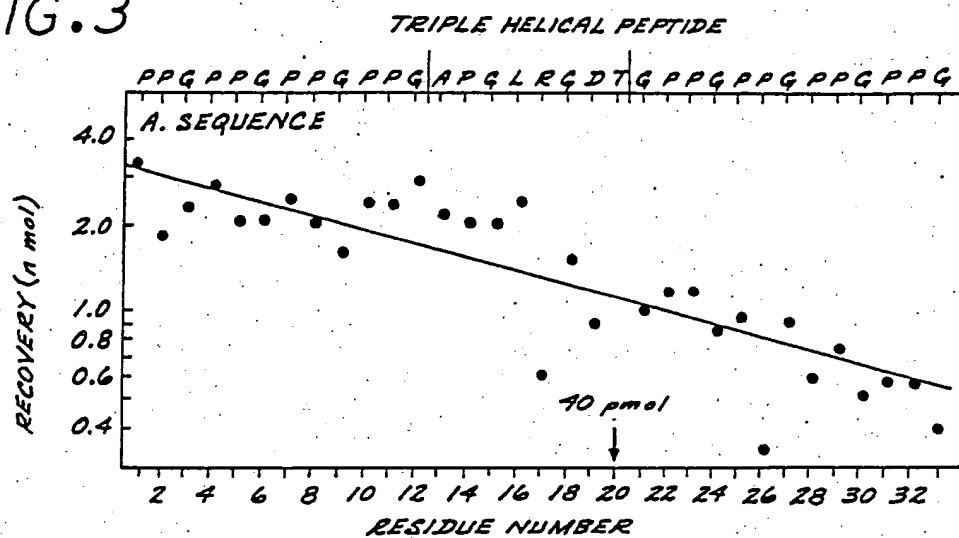
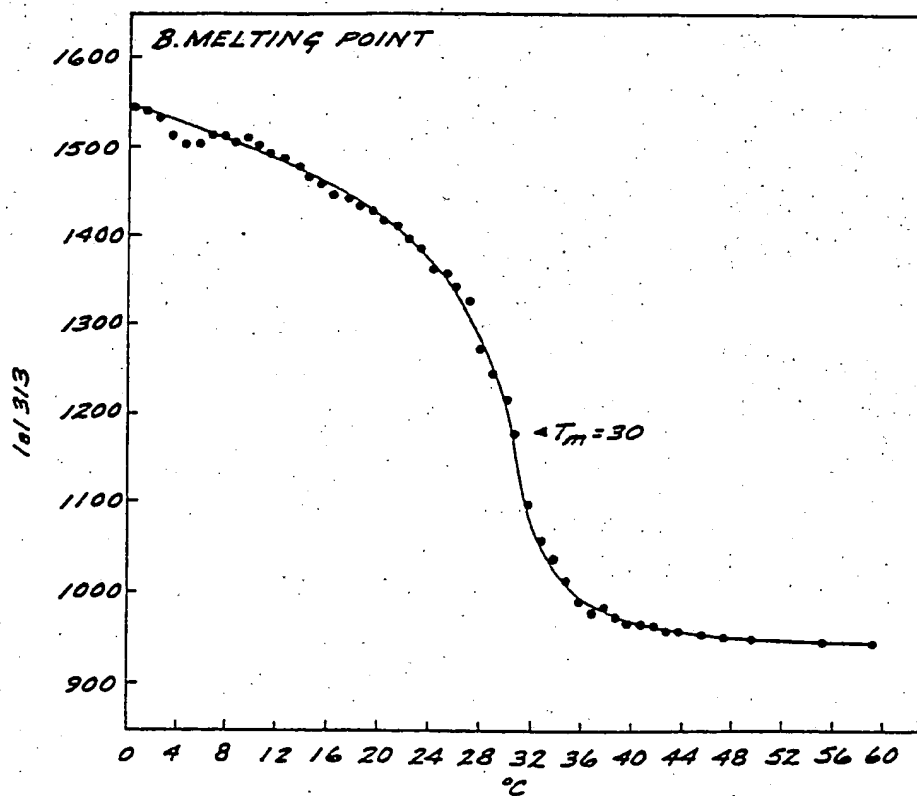
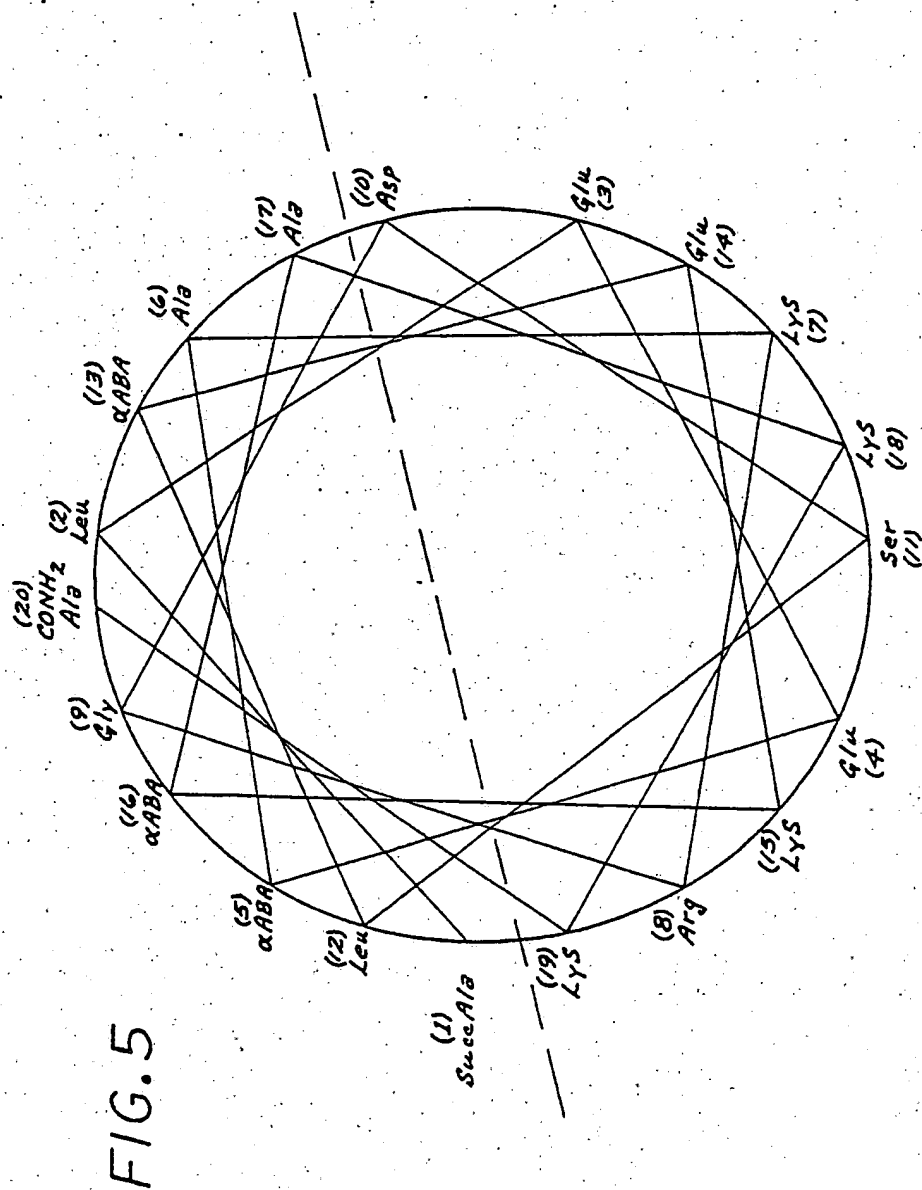


FIG. 4



3/4



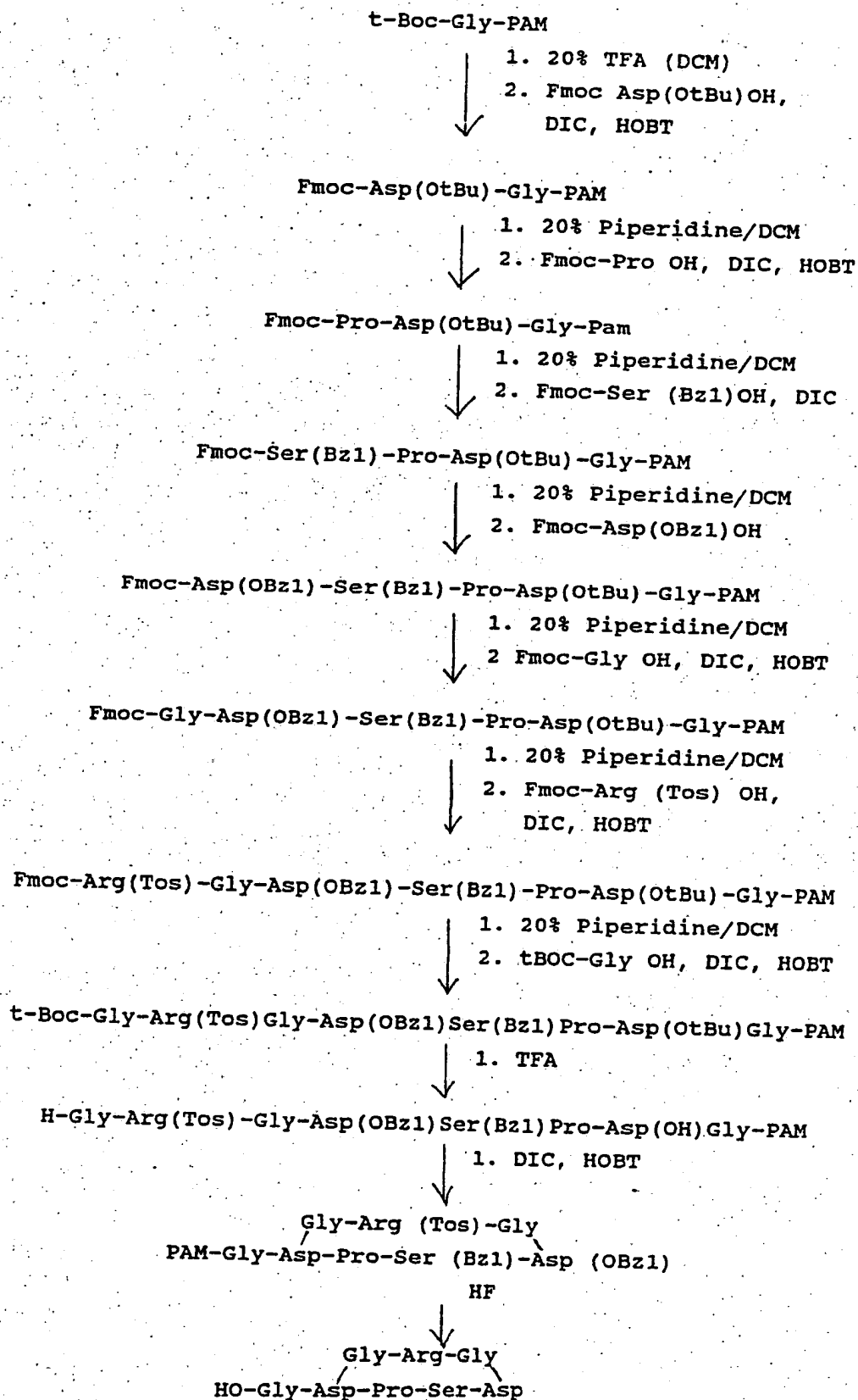


FIGURE 6

INTERNATIONAL SEARCH REPORT

International Application No PCT/US88/04403

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ¹ According to International Patent Classification (IPC) or to both National Classification and IPC INT. CL. 4th Ed. A61K 37/02; CO7K 5/12, 7/06, 08, 10 US. CL. 530/324, 325, 326, 327, 328, 329, 330; 514/12; 623/1, 2, 11, 66		
II. FIELDS SEARCHED Minimum Documentation Searched ⁴ Classification System Classification Symbols US 530/324, 325, 326, 327, 328, 329, 330; 514/12; 623/1, 2, 11, 66 Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁵		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴		
Category ⁶	Citation of Document, ¹⁰ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
X	Chemical Abstract, Vol. 104, Issued 26 May 1986 (Columbus, OH.) "Disialogangliosides GD2 and GD3 are involved in the attachment of human melanoma and neuroblastoma cells to extracellular matrix proteins". (Cheresh) abstract no. 183980c	1-44
X	Chemical Abstract, Vol. 107, Issued 06 July 1987 (Columbus, OH.) "Peptide inhibitors of fibronectin, laminin, and other adhesion molecules: unique and shared features". (Yamada) abstract no. 5066p	1-44
X	Chemical Abstract, Vol. 105, Issued 10 November 1986 (Columbus, OH.) "Antibodies and a synthetic peptide that block cell-fibronectin adhesion arrest neural crest cell migration in vivo." (Poole) abstract no. 169400c	1-44
* Special categories of cited documents: ¹⁵ "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step "Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "&" document member of the same patent family		
IV. CERTIFICATION Date of the Actual Completion of the International Search ² 02 March 1989 International Searching Authority ³ ISA/US Date of Mailing of this International Search Report ⁷ 04 MAY 1989 Signature of Authorized Officer ²⁰ DELBERT R. Phillips		

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

X	US,A, 4,589,881 (Pierschbacher) 20 May 1986 (see abstract).	1-44
X	US,A, 4,605,512 (Schaller) 12 August 1986 (See abstract).	1-44
X	US,A, 4,517,686 (Ruoslahti) 21 May 1985 (See abstract).	1-44
X,P	US,A, 4,789,734 (Pierschbacher) 06 December 1988 (See abstract).	1-44

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹⁰

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers because they relate to subject matter ¹² not required to be searched by this Authority, namely:
2. ☐ Claim numbers because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out ¹³, specifically:

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ¹¹

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No ¹⁸
X	The Journal of Cell Biology, Vol. 105, (No. 6, Pt. 2) Issued 1987 (Toda) "Fibronectin Receptors of Human Keratinocytes and Their Expression during Cell Culture". pages 3097-3104 (See abstract).	1-44
X	The Journal of Cell Biology, Vol. 104, Issued 1987, (Singer) "The Fibronectin Cell Attachment Sequence Arg-Gly-Asp-Ser Promotes Focal Contact Formation during Early Fibroblast Attachment and Spreading". pages 573-584 (See Abstract).	1-44
X	Developmental Biology, Vol. 123, Issued 1987 (Lash) "Synthetic Peptides That Mimic the Adhesive Recognition Signal of Fibro-nectin: Differential Effects on Cell-Cell and Cell-Substratum Adhesion in Embryonic Chick Cells". pages 411-420 (see summary).	1-44
X	The Journal of Biological Chemistry, Vol. 262, No. 36, Issued 25 December 1987, (Pierschbacher) "Influence of Stereochemistry of the Sequence Arg-Gly-Asp-Xaa on Binding Specificity in Cell Adhesion". pages 17294-17298 (See summary).	1-44
X	Blood, Issued 1985, Vol. 66, No. 4, (Haverstick) "Inhibition of Platelet Adhesion to Fibronectin, Fibrinogen, and von Willebrand Factor Substrates by a Synthetic Tetrapeptide Derived From the Cell-Binding Domain of Fibronectin". pages 946-952 (See Summary).	1-44
X	US,A, 4,578,079 (Ruoslahti) 25 March 1986 (See abstract).	1-44
X	US,A, 4,683,291 (Zimmerman) 28 July 1987 (See abstract).	1-44
X	US,A, 4,661,111 (Ruoslahti) 28 April 1987 (See abstract).	1-44
X	US,A, 4,614,517 (Ruoslahti) 30 September 1986 (See abstract).	1-44